Journal of Experimental Marine Biology and Ecology 498 (2018) 39–45

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Counteractive effects of increased temperature and pCO_2 on the thickness and chemistry of the carapace of juvenile blue crab, *Callinectes sapidus*, from the Patuxent River, Chesapeake Bay

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ARTICLE INFO

Keywords:
Blue crab
Callinectes sapidus
Carapace
Calcium and magnesium content
Climate change

ABSTRACT

Exoskeletons are central to the physiology and survival of marine invertebrates, but future increases in the temperature and pCO_2 of the marine environment may alter the biomineralization processes involved in their formation. Thus, it is important to consider the impacts of a changing climate on the functionality of invertebrate exoskeletons. In this study, juvenile blue crab, *Callinectes sapidus*, from the Chesapeake Bay were exposed to increased temperature and pCO_2 in a 2 × 2 factorial design for a period of two molts (approximately 30 days). Treatment levels were chosen to represent current (26 °C and 800 μatm CO_2) and predicted future conditions in the year 2100 (32 °C and 8000 μatm CO_2) in the Chesapeake Bay. Thickness was determined by light microscopy and carapace calcium (Ca) and magnesium (Mg) content were determined by Inductively Coupled Plasma – Atomic Emission Spectrometry. All Ca and Mg in the carapace were assumed to be present in the form of high-magnesium calcite (HMC). Increased temperature decreased the thickness of juvenile blue crab carapaces by 8.5% and significantly reduced weight percent HMC by 2.0% ($P < 0.05$). Increased pCO_2 significantly increased weight percent HMC by 2.0% but a significant increase in Mg content was also found. The observed counteractive effects of temperature and pCO_2 on weight percent HMC underscore the importance of assessing such interactions in studies that quantify the impacts of multiple environmental stressors. Combined with new data regarding the influence of increased temperature and pCO_2 on blue crab growth, the results of this study indicate tradeoffs between carapace thickness and chemistry with growth in juvenile blue crab exposed to future warming.

1. Introduction

Understanding the response of species to a changing climate is critical to determining the state of the marine environment in the future. The burning of fossil fuels has increased the amount of carbon dioxide in the atmosphere, and consequently the amount of dissolved carbon dioxide (pCO_2) in the ocean (Doney and Schimel, 2007). Recent IPCC scenarios predict that ocean pH will continue to decrease by 0.3 units and ocean surface temperatures will continue to increase by 2.6–4.8 °C by the year 2100 (Caldeira and Wickett, 2003; Orr et al., 2005; Stocker et al., 2013). These changes may especially impact marine invertebrates with exoskeletons that contain calcium carbonate. The exoskeleton is critical for protection from predators (Boßelmann et al., 2007), homeostasis of the internal environment (Roer and Dillaman, 1984), resistance to mechanical loads (Fabritius et al., 2012), attachment for musculature to facilitate swimming and gill movement in mobile marine invertebrates, and attachment to substrate for sedentary invertebrates (Smith and Chang, 2007; Vincent, 2002). Increases in temperature and pCO_2 may affect the invertebrate exoskeleton through changes in the availability of specific carbonate species for incorporation into the skeletal matrix and in the strength of ion gradients necessary for its formation.

Changes in the chemistry of the marine environment due to an increase in temperature and pCO_2 may affect carapace formation and functionality in a variety of ways. The functionality of the carapace is often quantified using calcium content and/or calcification rate. Previous work has identified increases (Long et al., 2013a; McDonald et al., 2009; Ries et al., 2009), decreases (Gazeau et al., 2007; Hoegh-Guldberg et al., 2007; Iglesias-Rodriguez et al., 2008; Lischka et al., 2010) and no effect (Coffey et al., 2017) of increased temperature and pCO_2 on carapace calcium content and/or calcification rate in a variety of marine calcifying species. Dissolution has been shown to be a secondary effect of zooxanthellae loss in corals (Anthony et al., 2008; Pandolfi et al., 2011) and mineral resorption to maintain hemolymph

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https://doi.org/10.1016/j.jembe.2017.11.005
Received 25 May 2017; Received in revised form 6 November 2017; Accepted 12 November 2017
Available online 20 November 2017

pH in mollusks (Gazeau et al., 2013), rather than direct dissolution due to external acidic conditions. The variability in the response of marine calcifiers to increased temperature and pCO_2 is related to the complexity of both the marine carbonate system and the calcification process itself. As pCO_2 increases, the saturation state of calcium carbonate will decline, moving towards a state in which dissolution of calcium carbonate is favored, creating a more adverse environment for shell formation (Gazeau et al., 2007). However, the nucleation of shell material from an internal calcifying fluid in many marine calcifiers (Cameron and Wood, 1985) may reduce the impact of changes in environmental chemistry on carapace formation. Nonetheless, the export of protons into a more acidic external environment may become thermodynamically unfavorable as ocean pCO_2 increases (Orr et al., 2005; Roer and Dillaman, 1984) and could impede the calcification process. The variability in the response to these competing forces described above underscores the importance of species-specific investigations of the impacts of climate change on marine calcifiers.

The blue crab, *Callinectes sapidus*, is a decapod crustacean found along the coast of the western Atlantic, from Massachusetts to Uruguay (Williams, 1984). It is a commercially and ecologically important species in the Chesapeake Bay (Hines, 2007; Hines et al., 1990; Miller et al., 2011). Blue crab are opportunistic, aggressive, benthic predators and their broad diet makes them an integral part of the benthic food web (Eggleston et al., 1992; Hines, 2007). Blue crab have been fished commercially in the Chesapeake Bay since the turn of the 19th century (Kennedy et al., 2007) and currently support important commercial and recreational fisheries in the region (Kennedy et al., 2007; Miller et al., 2011). The critical economic and ecological role of blue crab in the Chesapeake Bay estuary highlights the importance of quantifying the effects of future predicted increases in temperature and pCO_2 on this iconic species.

The exoskeletons of decapod crustaceans, such as blue crab, are complex biological structures which gain their strength from calcium carbonate, primarily present as high-magnesium calcite (HMC; Amato et al., 2008; Fabritius et al., 2012; Ries, 2011). Marine calcite often contains a substantial amount of magnesium (Mg) and is referred to as magnesian calcite, more specifically high-magnesium calcite (HMC) when the Mg content exceeds 3–4% (Mackenzie et al., 1983). Magnesian calcite is more soluble than pure calcite and its solubility generally increases with increasing Mg content (Berner, 1975). Increases in the molar Mg:Ca ratio of exoskeletons may be an indicator of reduced fitness and could be used to gauge the condition of animals exposed to external stressors, as has been suggested for other calcifying organisms, such as bivalves and foraminifera (Lorens and Bender, 1980; Toler et al., 2001). However, Mg content has also been associated with hardness of biogenic magnesian calcite (Kunitake et al., 2012) and may control calcite crystal morphology through the stabilization of amorphous forms of calcium carbonate during biogenic calcite formation (Loste et al., 2003).

The decapod crustacean exoskeleton is a structurally and chemically complex biological composite composed of HMC crystals with small amounts of amorphous calcium phosphate (Boßelmann et al., 2007; Ries, 2011; Roer and Dillaman, 1984). These crystals are incorporated into chitin-protein fibers that are arranged in a multi-layered structure (Chen et al., 2008; Raabe et al., 2005). Fabritius et al. (2012) used dispersive X-ray spectroscopy and confocal μ-Raman spectroscopy to characterize the structure and chemistry of the exoskeleton of the edible crab, *Cancer pagurus*. They determined that the external cuticle is mineralized with calcite, followed by a layer of amorphous calcium carbonate (ACC) containing magnesium-, phosphate-, and carbonate-rich phase, and finally an inner layer of ACC with magnesian calcite. However, there is little information on the structural and chemical complexity of blue crab carapaces. Ries (2011) determined that over 97% of the mineral phase of the newly acquired material in the carapaces of adult blue crab exposed to increased pCO_2 was HMC using X-ray diffraction.

The objective of our study was to examine the effect of increased water temperature and pCO_2 on the carapace thickness and chemistry of juvenile blue crab in the context of possible tradeoffs between crab growth dynamics and exoskeleton functionality in a changing climate. Glandon and Miller (2017) exposed juvenile blue crab to future levels of temperature and pCO_2 that are realistic for the Chesapeake Bay and found no effect of increased pCO_2 on growth and food consumption, despite positive responses to concurrent warming. Here, we examine the same specimens for any impact of these exposures on the chemistry and thickness of their exoskeletons.

2. Methods

2.1. Experimental setup and specimen selection

Specimens for carapace thickness and chemistry were obtained from animals exposed to increased water temperature and pCO_2, as described in Glandon and Miller (2017). Treatment levels were chosen to represent current summer conditions in the Chesapeake Bay (26 °C and 800 μatm) and predicted future conditions in the year 2100 (32 °C and 8000 μatm). In order to determine pCO_2 treatment targets for this study, data from the Chesapeake Bay Program Water Quality Database (CBPWQD) were used to determine the maximum pCO_2 values experienced in the Patuxent River from 2008 to 2012 (MdDNR, 2017). pCO_2 was calculated using pH, salinity, and temperature measured at three sites in the Patuxent River represented in the CBPWQD and total alkalinity (TA) measured in the experimental system during pilot experiments. The mean pCO_2 was 3267 ± 819 μatm (mean ± standard deviation) during this time period. There was a five-fold difference in the observed range of pCO_2 values; the average minimum pCO_2 was 1238 ± 775 μatm and the average maximum pCO_2 was 6364 ± 1611 μatm among all years and months sampled. Using these data, and in order to expose the crabs to the largest realistic range of pCO_2 values appropriate for the Chesapeake Bay system, 800 μatm was chosen as the low treatment level (just below the average minimum) and 8000 μatm was chosen as the high pCO_2 treatment level (just above the average maximum) for our experiment.

A flow-through experimental tank system was constructed using Patuxent River water at the Chesapeake Biological Laboratory. Juvenile blue crab (30–40 mm carapace width) were exposed to a 2 × 2 temperature × pCO_2 factorial experimental design for a period of two molts (27–39 days). Each treatment was replicated twice in each of two experimental blocks. Temperature was manipulated through a combination of heated and chilled seawater. pCO_2 was manipulated with the addition of CO_2 gas into aquaria mediated by a "pH-stat" system, whereby pH was lowered by a controller to pre-determined set points. At least 10 crabs were maintained individually at each treatment combination. Once each individual crab reached the inter-molt phase of the molt cycle (stage C4; Freeman et al., 1987) after its second molt, it was immediately frozen at − 20 °C for analysis. Carapaces from four crabs, selected at random per temperature × pCO_2 treatment per experimental block (total $n = 64$), were analyzed for both carapace thickness and concentrations of Ca and Mg.

2.2. Carapace thickness

Selected specimens were thawed and the dorsal carapace was fully removed from the remaining carcass. Dorsal carapace pieces were manually cleaned of any residual tissue and dried to constant weight at 60 °C. To minimize the effects of differences in thickness due to location on the carapace (Waugh et al., 2009), samples were taken from a standardized location on each crab. One-millimeter-square sections of the central portion of the dorsal carapace were removed for thickness analysis.

Following the methods of Waugh et al. (2009) and Secor et al. (1992), carapace sections were prepared for examination by light

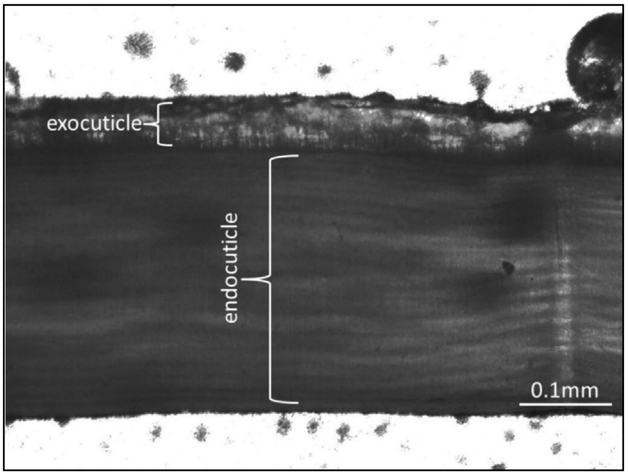

Fig. 1. Thin section of blue crab carapace, prepared using methods described in the text and viewed under light microscopy.

microscopy. Carapace sections were embedded in EpoFix cold-setting epoxy resin (Struers Inc., Cleveland, Ohio) blocks and allowed to cure for at least 24 h at room temperature. Epoxy blocks were then thin-sectioned (approximately 1.36 mm thick) using an Isomet low-speed diamond saw (Buehler, Lake Bluff, Illinois) and thin sections mounted onto a slide for further polishing. Thin section slides were polished through a series of three polishing papers: 320 grit, 1200 grit, and finally a microcloth fine polishing pad using alumina powder in order to obtain maximum clarity of the carapace section. Thin section slides were photographed under light microscopy (Olympus SZX9 microscope, Infinity1 camera) at 100 × magnification. The thickness of the entire cuticle section (mm) as well as the endocuticle layer was determined using ImageJ software (Rasband, 2012). The thickness of each thin section was measured at three different locations in order to account for the variability in thickness within a sample. The mean of the three measurements was used for analysis of exoskeleton layer thickness. Exocuticle thickness was determined as the difference between whole cuticle thickness and endocuticle thickness. Fig. 1 shows a section of carapace mounted and viewed under light microscopy, with the endocuticle and exocuticle layers clearly visible.

2.3. Ca and Mg analysis of the carapace

From each individual crab, about 200 mg of dried carapace was used for ICP-AES analysis. If a sufficient quantity of material was available, the area around the lateral spines was avoided, since that part of the carapace is often thicker than the medial dorsal area. However, in order to obtain 200 mg of sample, the entire dorsal carapace sometimes had to be used for chemical analysis. Each carapace sample was accurately weighed on a 5-digit analytical balance, then dissolved in 8 mL of 16 N nitric acid in a Milestone Ethos EZ microwave digester, in Teflon vessels with quartz inserts. A mixture of 30% H_2O_2 and Milli-Q water was placed in the Teflon vessels outside the inserts. Each vessel was linearly ramped to 200 °C in 10 min, then held at 200 °C for 10 min, and finally cooled under forced air for about 80 min until a safe temperature of < 50 °C was attained. Upon cooling, the contents of the quartz inserts were transferred to 30-mL Teflon vials and diluted 250-fold with Milli-Q water in 15-mL centrifuge tubes before analysis.

Concentration measurements were performed on the dilute solutions using a Perkin-Elmer Optima 8300 ICP-AES. Calibration standards were made gravimetrically in trace metal grade 2% nitric acid by combining certified standards of known Mg, Ca, and Sr concentrations in proportions to match those of a typical crab, as determined by a trial run using a standard method for measuring these elements in coral skeletal material (Schrag, 1999). Accuracy and precision were determined by measuring, alongside the crab samples, an in-house gravimetric standard solution and in-house coral solution with known Mg, Ca, and Sr concentrations that have been cross-calibrated by multiple laboratories using ICP-AES and Thermal Ionization Mass Spectrometry methods. Analytical precision was estimated to be ± 1.6% (1 sigma) relative standard deviation (RSD) for Ca and 4.6% (1 sigma) RSD for Mg, based on 30 determinations of the coral standard solution, with similar values obtained for the gravimetric standard. Accuracy was within 1% for both elements based on the same standards. The relatively high uncertainty for Mg was due to its low concentrations relative to Ca and instrument drift over the course of each run.

Elemental concentrations were expressed as mg/g carapace dry weight. Given the lack of information regarding the composition of blue crab carapaces, we expressed changes in carapace chemistry in terms of weight percent HMC, although we recognize this is a necessary simplification of the complex carapace structure. All Ca and Mg found in the carapace were taken to be present in the form of HMC, based upon the findings of Ries (2011) and consistent with an observed molar Mg:Ca ratio of HMC of at least 3–4% (7–9% in this study; Morse et al., 2006; Reeder, 1983). Measured concentrations were first converted to moles of Mg and Ca, and then to grams of $MgCO_3$ and $CaCO_3$ in the carapace, respectively. These weights were added and divided by the dry weight of the carapace to calculate the weight percent HMC. This calculation does not account for changes in the measured molar Mg:Ca ratio and whether or not Mg and Ca are present in a single carbonate phase, nor for the existence of other Mg- and/or Ca-bearing phases.

2.4. Statistical analyses

Data are presented as mean ± standard error by temperature × pCO_2 treatment. However, experimental data were analyzed as a full factorial design, with two levels of both temperature and pCO_2. All analyses were conducted in R (version 3.2.2 – R Core Team, 2015) using R-Studio (version 0.98.1103). An alpha level of $P < 0.05$ was used for all analyses. Analysis of Variance (ANOVA) using type II sum of squares was used to test for the effects of temperature, pCO_2, and their interaction on each response variable. In order to accurately represent the experimental design, temperature and pCO_2 were treated as fixed effects, and experimental block and tank were treated as random effects. Response variables included total carapace thickness (mm), endocuticle layer thickness (mm), exocuticle layer thickness (mm), Ca content (mg/g dry carapace), Mg content (mg/g dry carapace), weight percent HMC, and the molar Mg:Ca ratio. Significance of fixed effects and their interactions was assessed using the *Anova* function on linear, linear-mixed effects models using the *car* (Fox and Weisberg, 2010) and *nlme* packages (Pinheiro et al., 2015). When the analysis showed no significant interaction between the effects of temperature and pCO_2, the model was rerun without the interaction term in order to improve the power of tests of main effect.

3. Results

3.1. Carapace thickness

The carapace thicknesses of 64 individual blue crabs were determined for this study. Table 1 shows the sample size, mean, and standard deviation of the whole cuticle, endocuticle, and exocuticle thickness of the crabs by temperature × pCO_2 treatment. A two-way ANOVA indicated no significant temperature × pCO_2 interaction on whole cuticle, endocuticle, and exocuticle thickness, and therefore main effects of temperature and pCO_2 were considered ($F_{1,11} = 0.27$, 0.56, and 0.67, $P > 0.05$ for whole cuticle, endocuticle, and exocuticle thickness, respectively). The ANOVA indicated a marginally significant effect of temperature on whole cuticle thickness ($F_{1,12} = 3.83$,

Table 1
Sample size, mean, and standard error of the mean (SEM) of the whole cuticle, endocuticle, and exocuticle thickness (mm) of the crabs by temperature × pCO_2 treatment. Whole cuticle and endocuticle thickness were measured using image analysis software ImageJ. Exocuticle thickness was determined as the difference between whole cuticle and endocuticle thickness.

Temperature	pCO_2	*n*	Whole cuticle (mm)		Endocuticle (mm)		Exocuticle (mm)	
			Mean	SEM	Mean	SEM	Mean	SEM
Ambient	Ambient	16	0.32	0.01	0.27	0.01	0.056	0.003
	High	16	0.30	0.01	0.23	0.01	0.062	0.002
High	Ambient	16	0.29	0.02	0.24	0.01	0.053	0.002
	High	16	0.28	0.01	0.22	0.01	0.053	0.002

$P = 0.050$), but no significant effect of pCO_2 on whole cuticle thickness ($F_{1,12} = 2.44$, $P = 0.118$). The carapaces of crabs in high temperature water (0.28 ± 0.01 mm) were thinner than those of crabs in ambient temperature water (0.31 ± 0.01 mm), regardless of pCO_2 treatment. The ANOVA indicated that neither temperature nor pCO_2 had a significant effect on the thickness of the endocuticle or the exocuticle ($P > 0.05$ for all tests).

3.2. Carapace Ca and Mg content

The Ca and Mg content (mg/g dry carapace) of 64 individual crabs were determined for this study. Table 2 shows the number, mean, and standard error of the concentration of each element found in the carapace of blue crab by temperature × pCO_2 treatment. Weight percent HMC, calculated from the concentrations of Ca and Mg observed, and the molar Mg:Ca ratio, are also reported in Table 2.

A two-way ANOVA indicated no significant temperature × pCO_2 interaction ($F_{1,11} = 0.0053$, $P > 0.05$) on carapace Ca content, and therefore main effects of temperature and pCO_2 were considered. The ANOVA indicated a significant effect of temperature on Ca content; the carapaces of crabs in high temperature water contained significantly less Ca than those of crabs in ambient temperature water ($F_{1,12} = 14.06$, $P = 0.00016$; high temperature mean = 253 ± 2 mg/g, ambient temperature mean = 261 ± 2 mg/g). Fig. 2 shows the mean Ca content by temperature treatment. Although a trend of increasing Ca content was observed in carapaces of crabs exposed to high pCO_2 compared to those of crabs exposed to ambient pCO_2, the ANOVA indicated that it was not statistically significant ($F_{1,12} = 3.37$, $P = 0.064$).

A two-way ANOVA indicated a significant temperature × pCO_2 interaction on carapace Mg content ($F_{1,11} = 5.03$, $P = 0.025$), therefore pCO_2 effects were considered at each temperature level separately. The effect of increased pCO_2 was significant at both ambient and high temperature ($F_{1,11} = 8.61$ and 23.84, $P = 0.003$ and 1.05×10^{-6} for ambient and high temperature, respectively); the carapaces of crabs at high pCO_2 contained more Mg than those of crabs at ambient pCO_2, at both ambient and high temperature. However, the pCO_2 effect was greater at high temperature than at ambient temperature. The molar Mg:Ca ratio followed trends similar to the carapace Mg content. A two-way ANOVA indicated a significant temperature × pCO_2 interaction on the molar Mg:Ca ratio ($F_{1,11} = 4.53$, $P = 0.033$), hence pCO_2 effects were considered at each temperature level separately. The effect of increased pCO_2 was significant at both ambient and high temperature ($F_{1,11} = 5.38$ and 13.93, $P = 0.02$ and 0.00019 for ambient and high temperature, respectively); the carapaces of crabs at high pCO_2 had higher molar Mg:Ca ratios than those of crabs at ambient pCO_2, at both ambient and high temperature. However, the pCO_2 effect on the molar Mg:Ca ratio was greater at high temperature than at ambient temperature, as is clear from the interaction plot in Fig. 3.

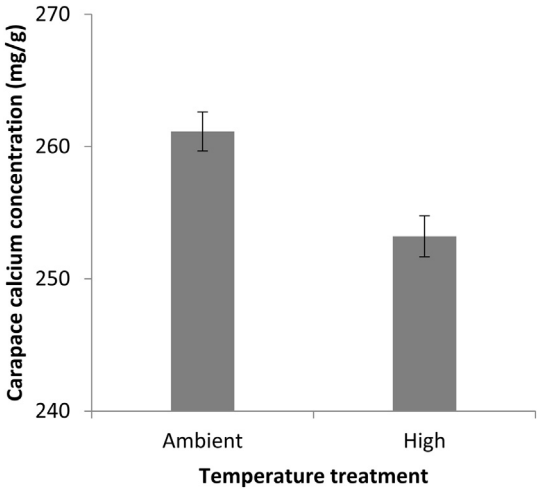

Fig. 2. Carapace Ca content (mg/g dry carapace) by temperature treatment. The carapaces of crabs exposed to high temperature water contained significantly less Ca than those of crabs exposed to ambient temperature water ($P = 0.00018$). There was no significant effect of increased pCO_2 on the Ca content of blue crab carapaces. Error bars represent the standard error of the mean.

A two-way ANOVA indicated no significant temperature × pCO_2 interaction ($F_{1,11} = 0.04$, $P > 0.05$) on carapace weight percent HMC, and therefore main effects of temperature and pCO_2 were considered. The ANOVA indicated a significant effect of temperature on weight percent HMC (Fig. 4); the carapaces of crabs in high temperature water had significantly lower weight percent HMC than those of crabs in ambient temperature water ($F_{1,12} = 7.18$, $P = 0.007$; high temperature mean = 67.5 ± 0.4%, ambient temperature mean = 69.0 ± 0.4%). Additionally, the ANOVA indicated a significant effect of pCO_2 on weight percent HMC (Fig. 4); the carapaces of juvenile crabs in high pCO_2 water had significantly higher weight percent HMC than those of crabs in ambient pCO_2 water ($F_{1,12} = 6.25$, $P = 0.012$; high pCO_2

Table 2
Sample size, mean (mg/g dry carapace) and standard error of the mean (SEM) of Ca and Mg content, weight percent HMC, and the molar Mg:Ca ratio found in the carapaces of juvenile blue crab by temperature × pCO_2 treatment.

Temperature	pCO_2	*n*	Ca (mg/g)		Mg (mg/g)		% HMC		Mg:Ca (mol:mol)	
			Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Ambient	Ambient	16	259	3	10.5	0.2	68.4	0.7	0.067	0.001
	High	16	263	1	11.2	0.2	69.6	0.3	0.070	0.001
High	Ambient	16	251	2	11.6	0.2	66.8	0.4	0.076	0.001
	High	16	255	3	13.2	0.3	68.3	0.7	0.085	0.002

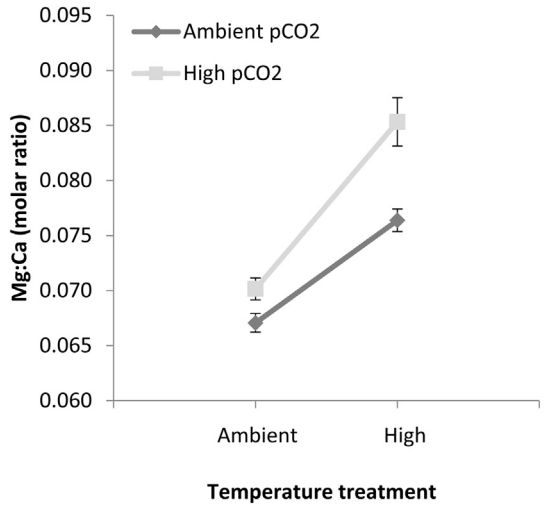

Fig. 3. Interaction plot of the molar Mg:Ca ratio in crab carapaces by temperature and pCO_2 treatment, showing a greater effect of pCO_2 treatment at high temperature than at ambient temperature (significant two-way ANOVA interaction; $P = 0.033$). At both temperature levels, the carapaces of crabs exposed to high pCO_2 had significantly higher molar Mg:Ca ratios than those of crabs exposed to ambient pCO_2 ($P = 0.02$ and 0.00019 for ambient and high temperature, respectively). Error bars represent the standard error of the mean.

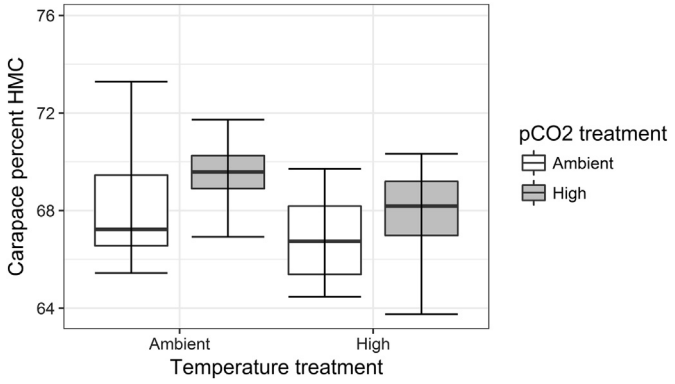

Fig. 4. Carapace percent high-magnesium calcite (HMC) by temperature and pCO_2 treatment. Solid dark line represents the median of each treatment combination, boxes represent inter-quartile range, and whiskers represent the sum of 1st (lower whisker) or 3rd (upper whisker) plus 1.5 times the inter-quartile range. There was a significant negative effect of increased temperature ($P = 0.007$) and significant positive effect of increased pCO_2 ($P = 0.012$) on carapace percent HMC.

mean = 68.9 ± 0.4%, ambient pCO_2 mean = 67.6 ± 0.4%).

4. Discussion

The data from this study reveal a counteractive effect of increased temperature and pCO_2 on the carapace chemistry of juvenile blue crab. Changes to the weight percent HMC can be manifested in our data in a variety of ways, each of which has a different potential effect on the crab carapace. Unlike the effect of carapace Ca content, which has been correlated to shell strength in a variety of invertebrate species (Amato et al., 2008; Fabritius et al., 2012; Jordaens et al., 2006), the effect of increased Mg content on the protective ability of the carapace is less clear. An increased proportion of Mg in the carapace may be an impediment to its formation due to the positive relationship between solubility and Mg content in magnesian calcite (Berner, 1975). Additionally, increases in the molar Mg:Ca ratio in the carapace may be an indicator of reduced fitness and could be used to gauge the condition of animals exposed to external stressors, as has been suggested for other calcifying organisms such as bivalves and foraminifera (Lorens and Bender, 1980; Toler et al., 2001). However, increased Mg content has been associated with increased hardness in biogenic calcites (Kunitake et al., 2012). Yet, there must be a balance between carapace thickness and strength, provided by Ca content, and flexibility, provided by Mg content, in order to provide protection while still allowing for growth through molting (Boßelmann et al., 2007; Roer and Dillaman, 1984). The data from this study indicate that increased temperature may cause a change in this balance through declines in carapace thickness and weight percent HMC, as well as changes to the molar Mg:Ca ratio of HMC in blue crab carapaces exposed to increased temperature.

Since the concentration of Ca is an order of magnitude higher than the concentration of Mg in our samples, changes to the Ca concentration have a much greater effect on the weight percent HMC than changes to the Mg concentration. This is evident in the effect of temperature on weight percent HMC in the crab carapaces. Increased temperature significantly reduced weight percent HMC in crab carapaces, which was caused by a decrease in the Ca concentration with temperature, despite an increase in Mg concentration. This represents a double negative effect of increased temperature on blue crab carapace chemistry, since Ca content declined as Mg content increased in the carapaces of the crabs exposed to increased temperature. On the other hand, Ca concentration was not affected by increased pCO_2, and this allowed for the relatively small increase in Mg content to result in a significant increase in weight percent HMC with increasing pCO_2. However, the overall effect of the observed differences in carapace chemistry by pCO_2 treatment is difficult to determine given the concurrent increases in weight percent HMC and Mg content in these samples.

The effects of increased temperature and pCO_2 on the carapace Mg:Ca ratio were synergistic, whereas the effects of increased temperature and pCO_2 on weight percent HMC were counteractive. The latter is evident in Table 2, where the mean weight percent HMC of the crabs exposed to ambient temperature and ambient pCO_2 is almost identical to the mean weight percent HMC of crabs exposed to high temperature and high pCO_2. In contrast, carapaces of crabs exposed to increased pCO_2 contained higher molar Mg:Ca ratios of regardless of temperature treatment, but the impact of increased pCO_2 was greater at high temperature than at ambient temperature (Fig. 3). Similar to the results from this study, non-linear responses have been observed in other species exposed to multiple climate stressors (e.g.: Crain et al., 2008; Dissanayake and Ishimatsu, 2011; McBryan et al., 2013).

The biological effects of the observed changes in blue crab carapace thickness and chemistry may be profound. Declines in carapace thickness and weight percent HMC have the potential to affect not only the ability of the carapace to protect the crab against predation, but also the process of carapace formation in this species. Increased pCO_2 causes a small increase in the bicarbonate concentration in seawater and may also affect the fractions of free Ca and Mg in seawater (Brezonik and Arnold, 2011). Since blue crab require these ions to create HMC in the carapace (Roer and Dillaman, 1984; Roer and Dillaman, 1993), acidification-related changes to the carbonate system may affect the carapace formation process in this species. However, the excellent ability of blue crab to regulate their internal chemistry (Henry and Kormanik, 1985; Mangum et al., 1985; Towle et al., 1976) may reduce the impact of changes in seawater chemistry on crab carapace thickness and composition since blue crab form the new carapace inside of the old one in a controlled environment (Cameron and Wood, 1985; Roer and Dillaman, 1984). Nonetheless, changes to the hydrogen gradient resulting from an increase in pCO_2 may impede the calcification process in blue crab. During the calcification process, protons are released during the creation of calcium carbonate from bicarbonate and Ca ions. Transport of these protons into a more acidic external environment may become thermodynamically unfavorable as ocean pCO_2 increases (Orr et al., 2005; Roer and Dillaman, 1984) and could represent an additional energy cost of carapace formation in an acidic environment. Hemolymph pH and crab metabolic rate were not quantified in this

Table 3
Summary of results of a two-way ANOVA to test the impact of increased temperature and pCO_2 on crab growth and consumption (shown in italics; from Glandon and Miller, 2017) and carapace thickness and chemistry. Alpha level for all tests was 0.05.

Response	Temperature	pCO_2
Growth rate	*Increase*	*No effect*
Carapace thickness	Marginal decrease	No effect
Carapace [Ca]	Decrease	No effect
Carapace [Mg][a]	Increase	Increase
Carapace % HMC	Decrease	Increase
Carapace Mg:Ca[a]	Increase	Increase

[a] Significant interaction between temperature and pCO_2 was observed.

study, but understanding the effects of increased environmental pCO_2 on these additional physiological parameters are an important next step in determining the effects of environmental change on blue crab.

Although the protection provided by a thick, heavily calcified carapace may contribute to the overall fitness of an individual crab, the creation of such a carapace is also energetically costly. The molting process in blue crab involves the active transport of various ions out of and into the cuticular space in order to allow for the formation of the new cuticle inside the animal while still maintaining optimal internal hemolymph chemistry (Roer and Dillaman, 1984; Roer and Dillaman, 1993). Therefore, tradeoffs may exist between the many energy-intensive processes that must occur after molting and the creation of an optimal exoskeleton. For example, in juvenile blue crab, rapid growth is prioritized for the crab to achieve a size refuge from predation as quickly as possible (Hines, 2007), possibly at the expense of carapace thickness or strength. Long et al. (2013b) observed a tradeoff between condition index and Ca content in red king crab (*Paralithodes camtschaticus*) and Tanner crab (*Chionoecetes bairdi*) exposed to acidic conditions. Reduced pH did not impact the Ca content of red king crab carapaces yet the condition index did decrease. Conversely, Tanner crab condition index remained unchanged while Ca content decreased when crabs were exposed to low pH conditions. The combination of the thickness and chemistry data with the growth data reported previously by Glandon and Miller (2017), indicate tradeoffs between juvenile blue crab growth and protective ability of the carapace in the face of a changing climate (Table 3). Glandon and Miller (2017) determined that juvenile blue crab exposed to increased temperature grew significantly faster than crabs exposed to ambient temperature conditions. The data reported here indicate a significant change in carapace chemistry in order to sustain growth when exposed to increased temperature. These data also suggest that while crabs can maintain growth rates similar to ambient conditions when exposed to increased pCO_2, the maintenance of growth is accompanied by an increase in weight percent HMC of the carapace. However, the increase in the molar Mg:Ca ratio in the carapaces of crabs exposed to increased pCO_2 suggests a more soluble material that could also be harder. The effect of an increase in the molar Mg:Ca ratio on the hardness or protective ability of the carapaces of crab exposed to increased pCO_2 remains to be investigated.

Impacts of environmental change and stress may be variable according to life history stage. The data from this study represent the juvenile life stage of blue crab, a time when rapid growth is prioritized in order to achieve a size refuge from predation (Hines, 2007). The importance of growth at the juvenile life stage is clear when considering the results of this study paired with the data from Glandon and Miller (2017). However, the data from this study do not speak to the impact of increased temperature and pCO_2 on the carapaces of adult blue crab; whether crabs held in conditions for many years would have more severe impacts, or if acclimation to those conditions would occur. Exposure studies over entire life histories are difficult and expensive to maintain, creating a dearth of data on acclimation response over a realistic period of time. Swiney et al. (2015) examined survival and development of Tanner crab, *Chionoecetes bairdi*, embryos from female crabs held in acidic conditions for two years. Oocyte and embryonic development, hatching success, and carapace Ca content were all negatively impacted as exposure to acidic conditions lengthened. Additionally, Long et al. (2016) observed a greater impact on Tanner crab larvae raised from adults held in acidic conditions than from larvae only exposed during the larval period, indicating significant carryover effects in this species. Recently, Coffey et al. (2017) observed no effect of pH or temperature on cuticle thickness in juvenile red and blue king crab exposed to low pH conditions for one year, despite elevated Ca content. Although Tanner, king, and blue crab are all from the infraorder Brachyura, few additional similarities exist between the species. Tanner and king crab are found in the Bering Sea and Gulf of Alaska while blue crab are found in the coastal and estuarine waters of the western Atlantic Ocean. Considering the large differences in habitat between the species, cross-species conclusions are difficult to make. Long-term studies of the effects of environmental change on estuarine crustaceans would greatly aid in understanding the success of these species in future climate conditions.

Understanding the effects of environmental variability on physiology is critical to predicting species response to future climate scenarios (Lefevre, 2016). Determining the relative importance of weight percent HMC and the molar Mg:Ca ratio of the carapaces of juvenile blue crab would help to quantify the biological significance of the observed changes in carapace composition in this study. Additionally, studies to quantify hardening time after the molt may shed light on how increased temperature and pCO_2 would impact recovery from molting. Any lengthening of the time to hardness could be detrimental to individuals, both as an increased energetic cost of time to recover and an increased risk of predation. Understanding how these effects may manifest through crab life history would be valuable to quantify the impact of changes in carapace thickness and chemistry at the population level. Finally, continuing to explore the secondary effects (e.g., metabolism, energy content) of increased temperature and pCO_2 on juvenile blue crab would help to paint a complete picture of the possible impact of climate change on this economically and ecologically valuable species.

Acknowledgements

We gratefully acknowledge A.W. Miller, K. Paynter, and J. Pierson for guidance throughout the project and comments on earlier versions of this article. J. Frank provided access to the ICP-AES for carapace analysis. Two anonymous reviewers greatly improved the presentation of this study. The assistance of E. Sheahan and A. Atkinson made the completion of the carapace thickness analyses possible. This is UMCES contribution number 5434. This work was supported by Maryland Sea Grant Coastal Resilience and Sustainability Fellowship to H.G.

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